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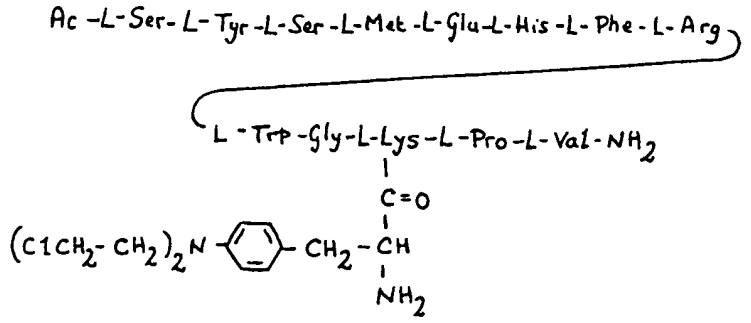
(54) Conjugate of alpha-melanotropin with p-L-sarcolysine (melphalan)

(57) The conjugate of alpha-melanotropin with 4-[bis(2-chloroethyl)amino]-L-phenylalanine has the formula



The conjugate and non-toxic, pharmaceutically acceptable acid addition salts thereof may used to treat malignant melanomas.

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SPECIFICATION

Conjugate of alpha–melanotropin with p–L–sarcolysine

The present invention relates to a new conjugate of alpha–melanotropin with 4–[bis(2–chloroethyl)amino]–L–phenylalanine and the non-toxic, pharmaceutically acceptable acid addition salts thereof, as well as to a process for the preparation thereof and to pharmaceutical compositions containing the new conjugate or acid addition salts thereof.

4–[Bis(2–chloroethyl)amino]–L–phenylalanine also called *p*–L–sarcolysine, has long been known as a chemotherapeutic agent for the treatment of various malignant tumors. However, this compound belongs to that group of cytotoxic compounds called "alkylating agents" which are characterized by their lack of selectivity towards tissues: not only do they attack malignant cells but also healthy cells. Thus, the use of this type of compound is not without danger to the organism. This generalized toxicity is particularly undesirable when the compound is used for the treatment of solid tumors in man. For this reason various solutions have been suggested in order to reduce the toxicity of the alkylating agents, while preserving their carcinolytic activity. In fact, all these suggestions are based on the same principle of linking *p*–L–sarcolysine by covalent bonding to other compounds of diverse nature and complexity which play the part of transporting the known alkylating agent specifically to the tumor cells.

Thus, it has already been proposed to link *p*–L–sarcolysine to peptides comprising 2 or 3 amino-acids in order to reduce the growth of Walker tumors in rats (see F. BERGEL and J.A. STOCK, J.Chem.Soc. (1960), 3658–3669).

U.S. Patent Specification No. 4,017,471 and Belgian Patent Specifications Nos. 838,273 and 880,970 describe cytotoxic agents obtained by coupling *p*–L–sarcolysine to an antibody or to an immunoglobulin specific for the tumoral antigens. However, the use of immunoglobulins is accompanied by undesirable side effects, such as pyrexia and anaphylactic shock, which can only be avoided by using an extremly pure immunoglobulin, i.e. by complete removal of the immunoglobulin fraction responsible for the side effects. However, obtaining an antitumor immunoglobulin having a high degree of purity necessitates the use of very elaborate methods of purification, such as for example affinity chromatography.

Finally, Belgian Patent Specification No. 775,775 describes synthetic oligopeptides containing 3–[bis(2–chloroethyl)amino]–L–phenylalanine (or *m*–L–sarcolysine) which can be used for treating certain neoplasms in man. These compounds would have an improved selectivity based on a specific inhibition of the incorporation of the amino-acids in the tumor cells but not in healthy cells. The recommended therapeutic form is, in fact, a mixture of six different oligopeptides called "Peptichemio". However, the therapeutic value of this product is unreliable. U. RINGBORG *et al.* have indicated in a recent article that they had not been able to find any difference of action between *m*–L–sarcolysine and peptichemio (see Eur.J.Cancer Clin. Oncol. *17*,(1981), 991–996). On the other hand, the clinical trials demonstrated that the administration of this product provokes numerous side effects, such as nausea, vomiting, phlebitis, alopecia and myeolophthisis (see M. CAVO *et al.*, Haematologica,*66*,(1981),208–215).

It is thus readily apparent that all of the solutions proposed hitherto do not, in practice, provide a drug which fulfils perfectly the purposes in view.

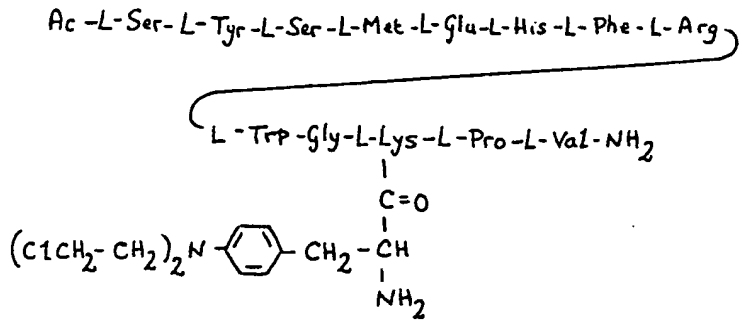
Malignant melanoma particularly affects fair-skinned individuals of the age group from 20 to 70 years. This tumor is especially the result of a cumulative and regular exposure to the sun. Its incidence has doubled and sometimes quadrupled in certain countries during the course of the last twenty years. It is one of the cancers which is most resistant to diverse therapies, such as surgical, chemotherapeutic, radiotherapeutic and even immunotherapeutic treatment. The best guarantee of being able to remedy this cancer appears to be, at the present time, its early diagnosis. Thus, it is imperative to search for routes providing new strategies of treatment.

Consequently, it appears to be very desirable to provide an anti-tumor drug which, while having an excellent cytotoxic activity, selectively attacks only malignant tumors of the melanoma type. Indeed, such a drug would constitute a remarkably effective therapeutic means for the treatment of malignant melanoma.

We have now discovered that by coupling *p*–L–sarcolysine specifically to alpha-melanotropin, it is possible to achieve fully this object.

Alpha-melanotropin is a well known natural hormone, the formula of which is that of an acetyltridecapeptide-amide. Its synthesis was carried out in 1963 (see R. SCHWYZER *et al.*, Helv.Chim.Acta,*46*,(1963), 870–889. Recent studies have shown that this hormone has the property of binding itself to the plasma membrane of certain types of tumor cells, more particularly to membrane receptors of certain human melanoma cell lines (see F. LEGROS *et al.*, Cancer Res.*41*,(1981),1539–1544).

Thus, according to the present invention, there is provided a new compound, i.e. a conjugate of alpha-melanotropin with 4–[bis(2–chloroethyl)amino]–L–phenylalanine of the formula:



(I)

as well as the non-toxic, pharmaceutically acceptable acid addition salts thereof.

In the compound of formula I, the sequence of 13 amino-acids having the L-configuration represents the structure of the natural hormone alpha-melanotropin or alpha-MSH (alpha-melanocyte-stimulating hormone), 4–[bis(2–chloroethyl)amino]–L–phenylalanine being linked by a covalent peptide bond to the free amino group at the epsilon position of the lysine present in the 11-position of alpha-melanotropin.

The compound of formula I has remarkable cytotoxic properties and, in particular, it is characterized by:

1) a marked selectivity with regard to certain malignant tumors of the melanoma type;

2) an intensity of action against such tumors which is 2 to 10 times better than that of *p*–L–sarcolysine itself. This increase of activity is quite surprising.

According to another aspect, in the compound according to the present invention, in contradistinction to the compounds of the prior art, *p*–L–sarcolysine is linked to a natural substance present in the human body which provides a far from negligible advantage having regard to the fact that the risk of appearance of undesirable side effects, for example anaphylactic shock, is clearly minimized.

According to the present invention, the conjugate of alpha-melanotropin with 4–[bis(2–chloroethyl)amino]–L–phenylalanine is prepared by a process in which an active ester of 4–[bis(2–chloroethyl)amino]–L–phenylalanine, the amino group of which is protected, is condensed with alpha-melanotropin, whereafter the protective group is removed from the alpha-melanotropin–4–[bis(2–chloroethyl)amino]–L–phenylalanine conjugate thus obtained.

4–[Bis(2–chloroethyl)amino]–L–phenylalanine used as starting material always has its amino group protected at the time of the condensation with alpha-melanotropin. As examples of protective groups to be attached in a reversible manner to the amino group of 4–[bis(2–chloroethyl)amino]–L–phenylalanine, there may be mentioned the *o*–nitrophenylsulfenyl, benzyloxycarbonyl, *p*–methoxybenzyloxycarbonyl, amyloxycarbonyl and biphenylisopropyloxycarbonyl groups and also preferably the *tert*–butoxycarbonyl group. However, there is no limitation of the choice of the protective group used, provided that this protective group can be easily removed from the amino group after condensation with alpha-melanotropin.

Furthermore, in order to facilitate its condensation with alpha-melanotropin, 4–[bis(2–chloroethyl)amino]–L–phenylalanine is converted into an active ester. As examples of compounds forming active esters, there may be mentioned l–hydroxybenzotriazole (HOBT), N–hydroxyphthalimide, l–hydroxypiperidine, 2–hydroxypyridine and N–hydroxysuccinimide, the N–hydroxysuccinimide ester of 4–[bis(2–chloroethyl)amino]–L–phenylalanine being preferred. The esterification of these compounds with 4–[bis(2–chloroethyl)amino]–L–phenylalanine may be carried out in known manner with the use of a coupling agent, such as dicyclohexylcarbodiimide (DCC), in an inert solvent, such as chloroform, dimethoxyethane or dimethylformamide and preferably in dichloromethane, at a temperature of from 0 to −10°C. during several hours.

The actual condensation of the active ester of 4–[bis(2–chloroethyl)amino]–L–phenylalanine, the amino group of which is protected, with alpha-melanotropin is generally carried out in the absence of light for a period of several hours at a temperature of from 18 to 25°C., preferably at ambient temperature, and in an inert solvent, such as dimethylformamide or dimethylacetamide, dimethylformamide being preferred.

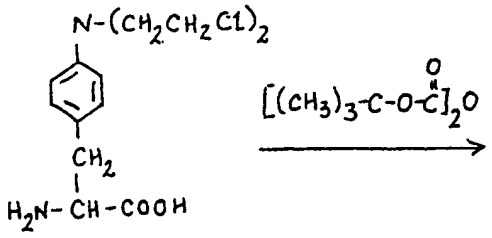
The molar ratio between the active ester of 4–[bis(2–chloroethyl)amino]–L–phenylalanine, the amino group of which is protected, and alpha-melanotropin is from 1:1 to 2:1 and preferably about 2:1.

At the end of the condensation reaction with alpha–melanotropin, the amino group of 4–[bis(2–chloroethyl)amino]–L–phenylalanine is still protected. In order to obtain the compound of formula I according to the present invention, it is necessary to remove the protective group from the conjugate of alpha-melanotropin with 4–[bis(2–chloroethyl)amino]–L–phenylalanine formed. This deprotection can be carried out by conventional methods, for example by hydro-

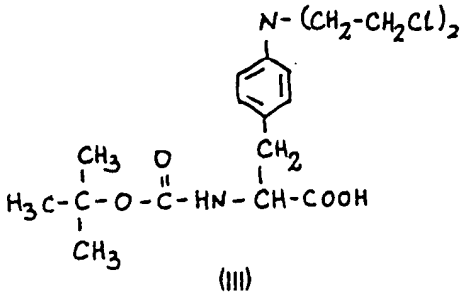
reaction is generally carried out at a temperatur of from 0 to 45°C., preferably at about 0°C., in the absence of light, in an inert solvent, such as dichloromethane or chloroform, and in the presence of a protective agent, such as 2–mercaptoethanol, methoxybenzene or 1,2–ethanedithiol and preferably methylthiobenzene.

When using the N–hydroxysuccinimide ester of 4–[bis(2–chloroethyl)amino]–L–phenylalanine, the amino group of which is protected by a *tert*–butoxycarbonyl radical, for the preparation of the new compound according to the present invention, the following reaction scheme explains the course of the reaction:

a) *Protection of the amino group.*

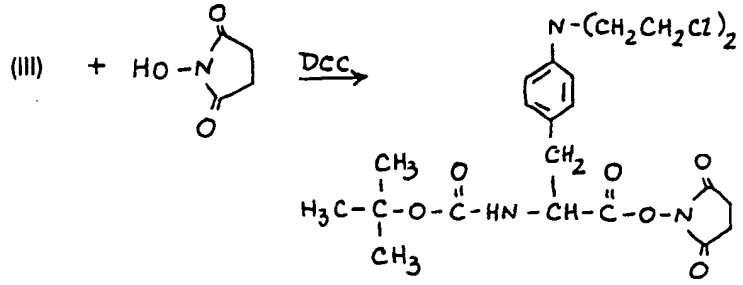


(II)



(III)

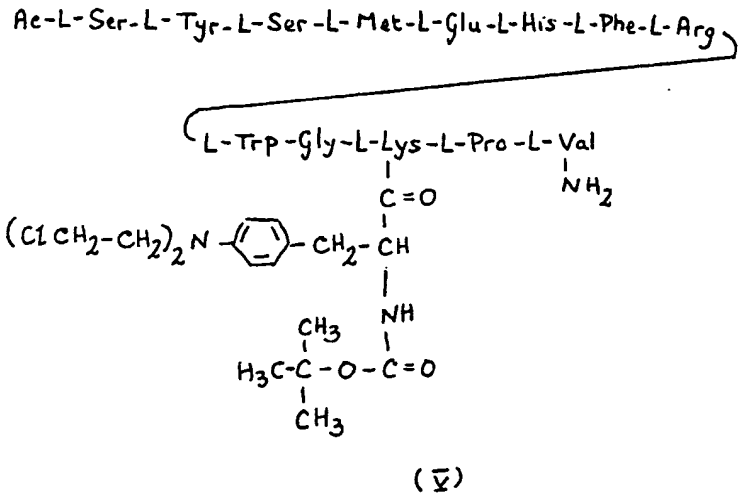
b) *Preparation of the active ester.*



(IV)

c) *Condensation (or coupling) with alpha-melanotropin.*

(IV) + alpha-melanotropine ⟶



(V)

d) *Deprotection.*

$$(V) \xrightarrow[\text{methylthiobenzene}]{CF_3COOH} (I)$$

The compound according to the present invention may be isolated as the free base or as a non-toxic, pharmaceutically acceptable acid addition salt thereof. The pharmaceutically acceptable acids used for this purpose may be inorganic acids, such as hydrochloric acid, or organic acids, such as acetic acid, trifluoroacetic acid or the like. The pharmaceutically acceptable acid addition salts may be prepared from the compound of formula I by known methods.

The following Example is given for the purpose of illustrating the present invention:

Example.

a) *Preparation of N–(tert–butoxycarbonyl)–4–[bis(2–chloroethyl)amino]–L–phenylalanine (formula III).*

3.7 g (12.09 mmoles) of 4–[bis(2–chloroethyl)amino]–L–phenylalanine are suspended in 140 ml of tetrahydrofuran containing 3.4 ml (24.184 mmoles) of triethylamine at a temperature of 0°C. and under a nitrogen atmosphere.

A solution of 3.958 g (18.138 mmoles) of di–*tert*–butyl carbonate in 60 ml of tetrahydrofuran is added dropwise thereto, followed by 30 ml of dimethylformamide in order to homogenize the reaction mixture.

The reaction mixture is stirred for 2 hours at 0°C. and thereafter overnight at ambient temperature. The solution is then evaporated under reduced pressure and the yellowish oily residue obtained is redissolved in 100 ml of a 5% w/v aqueous solution of sodium bicarbonate. This solution is extracted three times with 100 ml of petroleum ether and the aqueous phase is acidified to pH 3 with a IN hydrochloric acid solution.

The mixture is then extracted three times with 100 ml of ethyl acetate. The organic phases are dried over anhydrous sodium sulfate, filtered and then evaporated under reduced pressure. The residue is dissolved in 10 ml of a mixture of hexane, ethyl acetate and acetic acid (20:10:1 v/v/v). It is subsequently purified by chromatography on a silica column (LOBAR-MERCK silica 60 type C) using the same mixture as eluent (elution rate 60 ml/hour). There are thus obtained 4.16 g of N–(*tert*–butoxycarbonyl)–4–[bis(2–chloroethyl)amino]–L–phenylalanine in the form of a homogeneous oil. Yield: 85%.

Chromatography by TLC:

Rf=0.52 (hexane, ethyl acetate and acetic acid – 20:10:1 v/v/v)

0.85 (chloroform, methanol and acetic acid – 85:10:5 v/v/v).

The NMR spectrum of the compound obtained is in perfect concordance with that of the compound synthesized by K. HSIEH and G.R. MARSHALL (see J.Med.Chem.*24*,(1981),1304–1310).

b) *Preparation of the N–hydroxysuccinimide ester of N–(tert–*butoxycarb nyl)–4–[bis(2–chloroe-

0.657 g (5.71 mmoles) of N–hydroxysuccinimide is added to a solution containing 1.16 g (2.857 mmoles) of N–(*tert*–butoxycarbonyl)–4–[bis(2–chloroethyl)amino]–L–phenylalanine in 70 ml of dichloromethane. The solution is cooled to 0°C. and then a solution of 0.64 g (3.145 mmoles) of dicyclohexylcarbodiimide in 10 ml of dichloromethane is added dropwise thereto. The reaction mixture is then stirred for 2 hours at 0°C. and subsequently stirred overnight at ambient temperature. The precipitated dicyclohexylurea is filtered off and the filtrate is evaporated under reduced pressure.

The residue obtained is dissolved in a minimum amount of ethyl acetate from which a little dicyclohexylurea again precipitates. The solution is filtered and again evaporated. The residue is taken up in 100 ml of benzene and an oil separates out. This is removed by decanting and the benzene solution is then evaporated under reduced pressure. The residue obtained is dissolved in a minimum amount of benzene and the solution is added dropwise to 150 ml of petroleum ether cooled to 0°C. The white precipitate formed is filtered off, washed with a little petroleum ether and dried in a vacuum in the presence of sodium hydroxide.

There is thus isolated 1.43 g of the desired ester. This compound decomposes at 210–220°C. Yield: 95%, $[\alpha]_D^{25} = -26.4°$ (c=1, di–methylformamide).

Chromatography by TLC:

RF=0.44 (hexane, ethyl acetate and acetic acid – 20:10:1 v/v/v).

The absence of the free amino function in the molecule is demonstrated by the ninhydrin test (negative).

The amide bond is shown by the chlorine-toluidine reagent and the presence of the $N(CH_2CH_2Cl)_2$ radical is demonstrated by means of the colorimetric assay using 4–(*p*–nitrobenzyl)-pyridine (see K. HSIEH and G.R. MARSHALL, *loc. cit.*).

c) *Preparation of II–[N⁶–[N–(tert–butoxycarbonyl)–4–[bis(2–chloroethyl)amino]–L–phenylalanyl]–L–lysine]–alpha-melanotropin (formula V).*

2.2 g (1.32 mmole) of alpha-melanotropin are suspended in 45 ml of dimethylformamide. 1.326 g (2.64 mmoles) of the N–hydroxysuccinimide ester of N–(*tert*–butoxycarbonyl)–4–[bis(2–chloroethyl)–amino]–L–phenylalanine is then added thereto, together with 0.363 ml (2.64 mmoles) of triethylamine.

The suspension is stirred for 5 hours at ambient temperature and in the absence of light. There is thus obtained a yellow solution which is evaporated in vacuum (0.001 mbar). The residue obtained is taken up in a minimum volume of diethyl ether in which the product crystallizes. It is filtered off and washed three times with 50 ml of diethyl ether and then dried in vacuum in the presence of phosphorus pentoxide. There is thus obtained 1.928 g of II–[N⁶–[N–(*tert*–butoxycarbonyl)–4–[bis(2–chloroethyl)amino]–L–phenylalanyl]–L–lysine]–alpha-melanotropin in the form of a white powder. Yield: 71%. Decomposition at 210–220°C., $[\alpha]_D^{25} = -19.3°$ (c=1, dimethylformamide).

The product obtained is shown to be homogeneous by thin-layer chromatography, Rf=0.70 (*n*–butanol, pyridine, acetic acid and water – 4:1:1:2 v/v/v/v), and by high pressure liquid chromatography carried out under the following conditions:

HPLC column RP18–Merck, 10 micrometers, 4×250 mm.

eluent: triethylammonium formate of pH 3.5 (0.1% v/v aqueous solution) initially containing 30% of acetonitrile, the proportion of acetonitrile being progressively increased during the course of the elution to reach 50% after 40 minutes.

elution rate: 1.5 ml/minute.

detection: UV at 280 nm.

retention time of the compound obtained above: 22.7 minutes.

retention time of alpha-melanotropin: 6.2 minutes.

The absence of the free amino function in the molecule is shown by the ninhydrin test (negative). The presence of certain amino-acids in the molecule is shown by the following reagents: Sakaguchi (arginine), Pauly (tyrosine and histidine) and Ehrlich (tryptophane). The presence of the $N(CH_2CH_2Cl)_2$ radical is confirmed by means of the 4–(*p*–nitrobenzyl)pyridine reagent.

d). *Preparation of II–[N⁶–[4–[bis(2–chloroethyl)amino]–L–phenylalanyl]–L–lysine]–alpha–melanotropin.* (formula I).

1.928 g (0.94 mmole) of II–[N⁶–[N–(*tert*–butoxycarbonyl)–4–[bis(2–chlooroethyl)amino]–L–phenylalanyl]–L–lysine]–alpha–melanotropin is dissolved in 10 ml of a 50% solution of trifluoroacetic acid in dichloromethane containing 2% v/v of methylthiobenzene. The mixture is stirred at 0°C. in the absence of light for 30 minutes and then about 200 ml of diethyl ether are added. The white precipitate thus formed is filtered off, washed three times with 100 ml of diethyl ether and dried in vacuum in the presence of phosphorus pentoxide. There is thus obtained 1.891 g of the conjugate of alpha-melanotropin with 4–[bis(2–chloroethyl)amino]–L–phenylalanine which decomposes at 148–150°C.

Yield: 97%.

This compound contains 3 molecules of trifluoroacetic acid and 4 molecules of water per molecule. The compound is very hygroscopic. $[\alpha]_D^{25} = -25.2°$ (c=1, dimethylformamide).

The compound is shown to be homogeneous by thin-layer chromatography:
Rf=0.58 (*n*–butanol, pyridine, acetic acid and water – 4:1:1:2 v/v/v/v).
Analysis (M.W. of the hydrated salt: 2389) in %:

calc.:	F 7.1	$N(CH_2CH_2Cl)_2$ (+) 100	H_2O 3.0	
found:	6.6	96	3.2	

(+)=by colorimetric determination after reaction with 4–(*p*–nitrobenzyl)pyridine according to J. HSIEH and G.R. MARSHALL (*loc. cit.*).

Amount of anhydrous substance in the powder obtained: 82% (theory: 82.6%).

The presence of the free amino function is confirmed by the ninhydrin reagent and that of the $N(CH_2CH_2Cl)_2$ radical by the 4–(*p*–nitrobenzyl)pyridine reagent. The presence of certain amino-acids is also confirmed by specific reagents: Sakaguchi (arginine), Pauly (tyrosine and histidine) and Ehrlich (tryptophane).

The ratio of the amino-acids in the molecule is given in the following Table (analysis carried out by the method described by D.M. SPACKMAN, W.H. STEIN and S. MOORE, Anal.-Chem.30,(1958),1190).

Table I

Amino-acid	Relative composition	
	found	theoretical
serine	2.01	2
glutamic acid	1.04	1
proline	0.95	1
glycine	1.01	1
valine	1.00	1
methionine	0.94	1
tyrosine	0.98	1
phenylalanine	0.98	1
histidine	1.01	1
lysine	1.05	1
arginine	1.06	1
tryptophane (+)	-	1

(+) totally destroyed under the conditions of hydrolysis.

The product obtained still contains some small impurities as shown by high pressure liquid chromatography under the conditions indicated in the preceding step.

This product may be purified further to a degree of purity exceeding 99% by liquid chromatography on a silica column (Lobar-Merck – silica 60 – type C) (eluent:*n*–butanol, pyridine, acetic acid, water 4:1:1:2 v/v/v/v; elution rate 60 ml/hour).

The fractions containing the product are evaporated under reduced pressure. The residue is partly dissolved in 200 ml of distilled water. After lyophilization, there is obtained a white powder which only shows a single peak by high pressure liquid chromatography (HPLC).

Pharmacological results

The new compound according to the present invention was subjected to pharmacological tests and gave the results set out hereinafter.

1) *Cytotoxicity in vitro.*

This test is based on the measure of the extent of the incorporation into different tumor cell lines of precursors of the synthesis of DNA, such as [^{3}H]thymidine, after the cells have been exposed to the action of the compounds to be tested. The more the receptor sites of the tumor

[^{3}H]thymidine will occur, so that the synthesis of DNA in the cell will slow down. This test gives an indirect measure of the cytotoxicity of the compounds tested.

The incorporation of [^{3}H]thymidine was studied on the following melanoma cell lines: HM6 (human), B16 (mouse), ME67 (human), SCL (human) and HBL (human) as well as on human osteosarcoma OSC–1B, this last line being non-receptive to alpha-melanotropin.

The compound according to the present invention (compound A) and *p*–L–sarcolysine (compound B) used as a reference compound are prepared in MEM (minimum essential medium) (Gibco Biocult, Glasgow, Great Britain). For this purpose, the compounds are first dissolved in the HC1/ethanol solvent specially provided for this use (provided by Welcome C°); then serial dilutions of the parent solution by means of MEM (additionally containing 20% of fetal bovine serum (FBS, Gibco Biocult) and 10 mmoles of glutamine per 100 ml), allow adjustment of the concentrations to doses of 1, 5, 10, 50 and 100 µg/ml of the compounds to be tested.

The technique used is described by A. VERCAMMEN-GRANDJEAN *et al.* in Anticancer Research,*2*,(1982),133–140).

The melanoma cells are cultured in a monolayer in Nunc wells in Ham's F–10 medium supplemented with 10% FBS (Gibco Biocult).

The osteosarcoma cells OSC–1B are cultured in RPMI culture medium (Roswell Park Memorial Institute; Gibco Biocult) containing 10% FBS and 100 units/ml of a mixture of penicillin and streptomycin. The cells are incubated for 1 hour at 37°C. in the presence of the compound to be tested and then for 2 hours with [^{3}H]thymidine. They are then washed three times with a phosphate-buffered physiological salt solution (potassium chloride, 200 mg; monopotassium dihydrogen phosphate, 200 mg; calcium chloride dihydrate, 132 mg; magnesium chloride hexahydrate, 100 mg; disodium monohydrogen phosphate dihydrate, 1150 mg; sodium chloride, 8000 mg and distilled water, 1000 mg; pH 6.8) and lyzed by 0.2 ml of a 1N solution of sodium hydroxide. The radioactivity of this volume (0.2 ml) is measured with the help of a scintillation counter.

The results obtained are given in the following Table II and are expressed as a percentage of the incorporation of the [^{3}H] thymidine in the cells which have not been cultured in the presence of the test compounds (control). The values indicated in the first column of this Table are the doses in µg/ml used for each of the compounds A and B; the values indicated between brackets designate the corresponding dose of *p*–L–sarcolysine present in compound A.

Table II

Dose in µg/ml	Incorporation (in %) of ^{3}H-thymidine into the cell lines											
	HM6		B16		ME67		SCL		HBL		OSC-1B	
	A	B	A	B	A	B	A	B	A	B	A	B
1 (0.15)	100	97	125	120	125	125	80	110	95	90	110	90
5 (0.77)	120	80	125	120	90	105	65	90	n.d.	n.d.	n.d.	n.d.
10 (1.54)	85	65	105	95	80	n.d.	65	85	73	93	90	70
50 (7.70)	10	60	45	95	70	85	15	65	53	75	n.d.	n.d.
100 (15.40)	5	50	45	90	65	85	15	25	45	70	60	40

n.d.=not determined

Table II shows, on the one hand, the specificity of action of compound A (compound according to the present invention) with regard to melanomas, i.e. cells receptive to alpha-melanotropin. On the other hand, it also shows that, at a dose of from 50 to 100 µg/ml, compound A possesses a cytotoxic activity which is higher than that of *p*–L–sarcolysine (compound B) by a factor of 2 to 10, according to the cell lines.

Moreover, taking into account that the ratio between the molecular weight of compound A in the form of the anhydrous base and that of compound B is equal to 6.5, it can be deduced that the observed increase by a factor of 10 is already obtained with a dose of compound A which, when expressed as a molar quantity, is 6.5 lower than that of compound B.

2) *Biodistribution.*

The biodistribution of compound A, labeled with iodine 125, was studied in mice C–57 BL carrying melanoma B16. The compound was administered intravenously into the tail of the animal at a dose of 1 ng/0.4 ml (corresponding to a radioactivity of 2×10^6 counts per minute or

1.1 μCi/0.4 ml).

The following Table III gives the activity accumulated in different organs at the moment when the concentration of the product had reached its maximum, i.e. about 20 minutes after injection. The activity is expressed as a percentage of the total activity corresponding to the dose administered.

Table III

Organs	Average moist weight (mg)	Activity (%) (in constant weight)
liver	900	5
kidneys	200	5
skin	200	3
tumor	150	2
stomach	100	1.5
pancreas	-	1.5
lungs	-	1.5
submaxillary glands	50	1
intestines	150	0.8
muscle	200	0.7
thyroid	-	0.6
tongue	80	0.6
spleen	-	0.5
fat	55	0.4
heart	-	0.3
thymus	-	0.3
bone	-	0.25
brain	-	0.2
ovaries	-	0.08
suprarenals	-	0.04
hypophysis	-	0.02

Table III indicates that an important amount of the labeled substance is found in the tumor, thus confirming the privileged recognition thereof for the compound of the present invention.

3) *Alpha-melanotropin receptor binding assay.*

This test allows to determine whether a given compound competes with alpha-melanotropin, labeled with iodine 125, for binding to the receptor sites on the melanoma cells. Thus, it can be used to demonstrate that the compound of the present invention (compound A) recognizes the same receptors on the melanoma cells as alpha-melanotropin.

For this purpose, melanoma cells are incubated in the presence of [^{125}I]–alpha-melanotropin and in the presence of increasing amounts of the compound to be tested. If the latter attaches to the same receptors sites as the labeled compound, the amount of [^{125}I]–alpha-melanotropin incorporated into the melanoma cells decreases when the concentration of the test compound increases. The measure of the amount of labeled alpha-melanotropin incorporated into the melanoma cells then allows to determine the degree of affinity of the test compound for the receptor sites of alpha-melanotropin.

In a typical experiment, 10^6 cells of the human melanoma cell line UPL are cultured in 100 µl

mmoles of glutamine. The cells are incubated for 15 minutes at 37°C in the presence of 100 µl of RPMI culture medium containing [^{125}I]–alpha-melanotropin (corresponding to a radioactivity of 10^6 counts per minute or 1, 500 µCi/µg), and in the presence of 100 µl of the aforesaid RPMI culture medium containing increasing amounts of the compound to be tested. The cells are then washed three times with a cold buffered physiological salt solution and the cellular material is separated by a 1 minute centrifugation at 3,000 g. The radioactivity of the isolated cellular material is measured.

In this test, the compound according to the present invention (compound A) is compared with *p*–L–sarcolysine (compound B), unlabeled alpha-melanotropin (natural alpha-MSH) and an equimolar mixture of unlabeled alpha-melanotropin and *p*–L–sarcolysine, respectively.

The results obtained are given in the following Table IV; they are expressed as the percentage of the radioactivity obtained when incubating the melanoma cells with the labeled alpha-melanotropin only.

TABLE IV

Incorporation (in %) of [^{125}I]-alpha-melanotropin into human HBL melanoma cells.

molar concentration	Test compounds			
	A	B	α-MSH	α-MSH + B
0	100	100	100	100
10^{-11}	68	98	90	90
10^{-10}	52	96	82	83
10^{-9}	44	95	62	68
10^{-8}	37	88	42	44
10^{-7}	30	90	28	30
10^{-6}	28	95	22	24

Table IV shows clearly that compound A competes with the labeled alpha-melanotropin for the recognition of the same receptor sites on the melanoma cells. It indicates further that the affinity of compound A for the receptor sites is higher than that of alpha-melanotropin (alpha–MSH) at a constant molar concentration. It can be seen also that the affinity obtained for a concentration of 10^{-9} mole of alpha–MSH can already be attained for a concentration of 10^{-11} mole of compound A, i.e. a concentration which is 100 times lower.

On the other hand, *p*–L–sarcolysine (compound B) has no affinity whatsoever for the receptor sites of alpha–melanotropin. Furthermore, when comparing the results obtained with compound A and with the equimolar mixture of alpha-melanotropin and *p*–L–sarcolysine, it can be seen that the affinity of the compound of the present invention (compound A) is greater than the affinity of the mixture.

Moreover, it has been proved hereinabove (Table II) that this greater affinity of compound A for the receptor sites of alpha-melanotropin is also accompanied with a markedly superior cytotoxic activity, when compared with *p*–L–sarcolysine.

The new compound according to the present invention, as well as the non-toxic pharmaceutically acceptable acid addition salts thereof, are useful for the treatment of malignant melanoma in the form of pharmaceutical compositions containing these compounds as active ingredients together with solid or liquid pharmaceutical carriers or excipients.

The pharmaceutical carriers or excipients used in these compositions are well known in the art. They may be, for example, water, gelatine, lactose, starch, magnesium stearate, talc, sodium bicarbonate, citric acid, polyvinylpyrrolidone, sodium benzoate and the like, as well as other excipients currently used for this purpose.

These pharmaceutical compositions can be administered intravenously, intraperitoneally, intramuscularly or subcutaneously and also per os, the dosage administered varying with the mode of administration, the severity and the stage of development of the tumors to be treated.

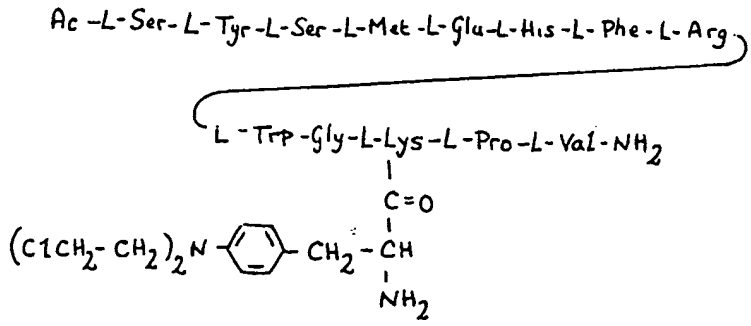
A particularly preferred mode of administration for the treatment of malignant melanoma located on a limb of a patient, consists of introducing a solution of the compound according to the present invention into the circuit used for the extracorporeal blood circulation of the bad limb.

For this purpose, the blood circulation of the bad limb is isolated from the general blood circulation, and the blood circulating in the limb and in the extracorporeal circuit is diluted with an adequate volume of Hartman solution ($CaCl_2.2H_2O$, 18–22 mg; KCl, 27–33 mg; NaCl, 570–630 mg; sodium lactate, 290–330 mg; water for injection, 100 ml) so that the final concentration of the total proteins present in the diluted blood is lower than 100 mg/dl. Thereafter, the solution of the compound of the present invention is introduced into this circuit.

In this mode of administration, there is used advantageously a dose of from 200 to 800 mg of the compound according to the present invention, dissolved in 200 to 800 ml of an aqueous solution of hydrochloric acid, the pH of which is between 3 and 4. This solution is injected into the circuit of the extracorporeal circulation in 4 to 5 minutes and the extracorporeal circulation is maintained for 30 minutes.

CLAIMS

1. A conjugate of alpha-melanotropin with 4–[bis(2–chloroethyl)amino]–L–phenylalanine of the formula



and the non-toxic, pharmaceutically acceptable acid addition salts thereof.

2. A process for the preparation of the conjugate according to claim 1, which comprises condensing an active ester of 4–[bis(2–chloroethyl)amino]–L–phenylalanine, the amino group of which is protected, with alpha-melanotropin and removing the protective group from the alpha-melanotropin–4–[bis(chloroethyl)amino]–L–phenylalanine conjugate thus obtained.

3. A process according to claim 2, wherein the active ester of 4–[bis(2–chloroethyl)amino]–L–phenylalanine is an ester of l–hydroxybenzotriazole, N–hydroxyphthalimide, l–hydroxypiperidine, 2–hydroxypyridine or N–hydroxysuccinimide.

4. A process according to claim 2 or 3, wherein the amino group of the 4–[bis(2–chloroethyl)amino]–L–phenylalanine is protected with a protective group selected from o–nitrophenylsulfenyl, benzyloxycarbonyl, *p*–methoxybenzyloxycarbonyl, amyloxycarbonyl, biphenylisopropyloxycarbonyl and *tert.*–butoxycarbonyl groups.

5. A process according to any of claims 2 to 4, wherein the condensation of the active ester of 4–[bis–(2–chloroethyl)amino]–L–phenylalanine, the amino group of which is protected, with alpha-melanotropin is carried out in the absence of light for a period of several hours at a temperature of from 18 to 25°C. and in an inert solvent.

6. A process according to any of claims 2 to 5, wherein the molar ratio of the active ester of 4–[bis–(2–chloroethyl)amino]–L–phenylalanine, the amino group of which is protected, to alpha-melanotropin is from 1:1 to 2:1.

7. A process according to any of claims 2 to 6, wherein the removal of the protective group from the alpha-melanotropin–4–[bis(2–chloroethyl)amino]–L–phenylalanine conjugate is effected by hydrolysis in an acidic medium at a temperature of from 0 to 45°C. in the absence of light, in an inert solvent and in the presence of a protective agent.

8. A process according to claim 7, wherein the acidic medium in which the hydrolysis is carried out is trifluoroacetic acid.

9. A process for the preparation of the conjugate according to claim 1, substantially as hereinbefore described and exemplified.

10. The conjugate according to claim 1, whenever prepared by the process according to any of claims 2 to 9.

11. A pharmaceutical composition comprising a therapeutically effective amount of a conju-

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